# The Impact of DDR1 on Mechanics and Mineralization of Mice Femora

THESIS

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By

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## Abstract

Collagen type I is the most abundant extracellular matrix protein and the major structural component of connective tissues found in the human body. Collagen fibrils play a critical role in the mineralization and mechanics of bone. The collagen fibril structure can be modified by certain non-collagenous proteins (NCP) which bind to collagen, such as the discoidin domain receptor (DDR1). Understanding the role collagen fibrils play in bone mineralization is relevant to the field of biomaterials as well as in understanding the pathogenesis of diseases characterized by aberrant mineralization. The NCP DDR1 has been shown to impact the structure of collagen, but the impact that this structural change has on bone mineralization and mechanics has yet to be determined.

The Agarwal lab has previously established that by binding to collagen and disorganizing the fibril structure, the collagen receptor DDR1 modulates collagen fibrillogenesis and the resulting morphology of collagen fibrils in both in-vitro and in cell-based assays. This project aims to further the field by analyzing how mineralization in collagen fibrils is differentially impacted by the presence of DDR1 in the femora of murine models. Studies were conducted on the femora extracted from six month old DDR1 Knout-out (KO) and Wild-type (WT) female and male mice. Mechanical properties of the femora, such as stiffness, were determined from 3-point-bending experiments and the resulting load vs. displacement data. The broken bone samples were then cleaned and defatted and subjected to Thermogravimetric analysis (TGA) and Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) protocols to

determine mineral content and relative weight percentages of Calcium (Ca) and Phosphorous (P) respectively.

Consistent gender specific trends were seen in the collected biomechanical and mineral data. In female mice, a statistically significant difference was observed with the DDR1 KO mice femurs showing a higher stiffness than WT mice femurs. The higher stiffness that was found in female KO mice was consistent with the higher mineral content found in female KO mice compared to their WT litermates. An opposite trend was seen in male mice, although the results were not significant. These results show how DDR1 influences bone mechanics and mineralization in a gender specific manner.

## Acknowledgments

I would like to thank my Research Advisor, Dr. Gunjan Agarwal, for her guidance and support during my entire undergraduate research experience. Dr. Agarwal provided me with great opportunities in her lab and has been a great encouragement throughout this entire thesis project. My experiences in her lab and this thesis project have taught me how to conduct independent and productive work, especially in the field of research. I am grateful for all of the opportunities and for such a rewarding research experience.

I would also like to thank Dr. Alan Litsky and Dr. Nicole Karn for collaborating on this research project. Their expertise in their respective fields and their willingness to provide of their time and resources made the completion of this project possible. Thank you very much for the crucial parts you both played in this research project.

I would also like to thank all of my lab peers and colleagues in the Agarwal Lab for their mentorship, advice, and camaraderie: David Yeung, Brook Ollander, Arghavan Farzadi, Blain Jones, and Rachel Novinc. It has been wonderful to work with you all and an even greater pleasure getting to know everyone over this last year.

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# Introduction

Fibrils of collagen type I serve as the biological template for bio-mineralization in bone tissue, with calcium phosphate minerals in the form of carbonated hydroxyapatite (HA) being the major inorganic component[1]. Bone tissue composite is comprised of a hierarchical structure consisting mostly of an organized collagen fibril scaffold with nanometer sized crystals of hydroxyapatite[2]. The biomechanics of bone tissue have been extensively studied[3,4] with recent computer simulations illustrating how the mineral component of bone is primarily responsible for bearing the stress of loads and how the collagen fibrils are responsible for bone's deformation response[2]. The process of mineralization in bone tissues has been shown to be dependent on the collagen fibril structure and the presence of non-collagenous proteins (NCPs) in in-vitro studies[5,6]

*Collagen Fibril Structure and Mineralization*

The Collagen fibril is formed through the self-assembly of individual triple helical collagen molecules[7]. Collagen fibrils are characterized by a 67 nm periodicity in their structure that can be seen in electron microscopy images as distinct striations referred to as the D-period[7]. These striations are associated with how the individual collagen molecules assemble in a staggered formation along the axial direction[7]. The D-period also consists of 40 nm gap zones that have been associated with the nucleation of apatite crystals [5]. Computer simulations have illustrated how the charged amino acids that lie in the gap regions are crucially responsible for the promotion of apatite formation in the

intrafibrillar mineralization of collagen[6]. Figures 1 and 2 below detail the periodicity, hierarchy, and organization of the collagen structure.

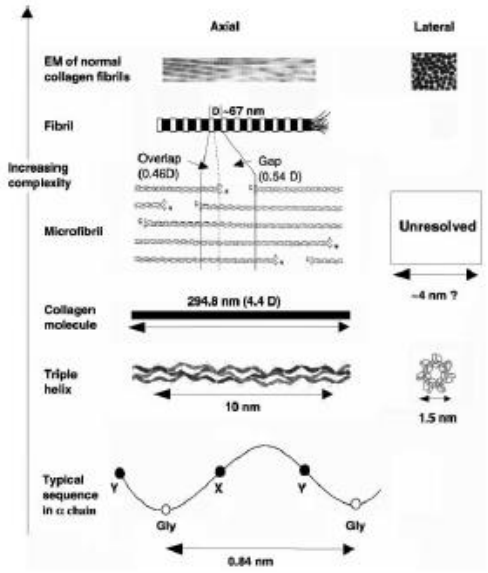



**Figure 1:** Hierarchy of Collagen Structure[7].

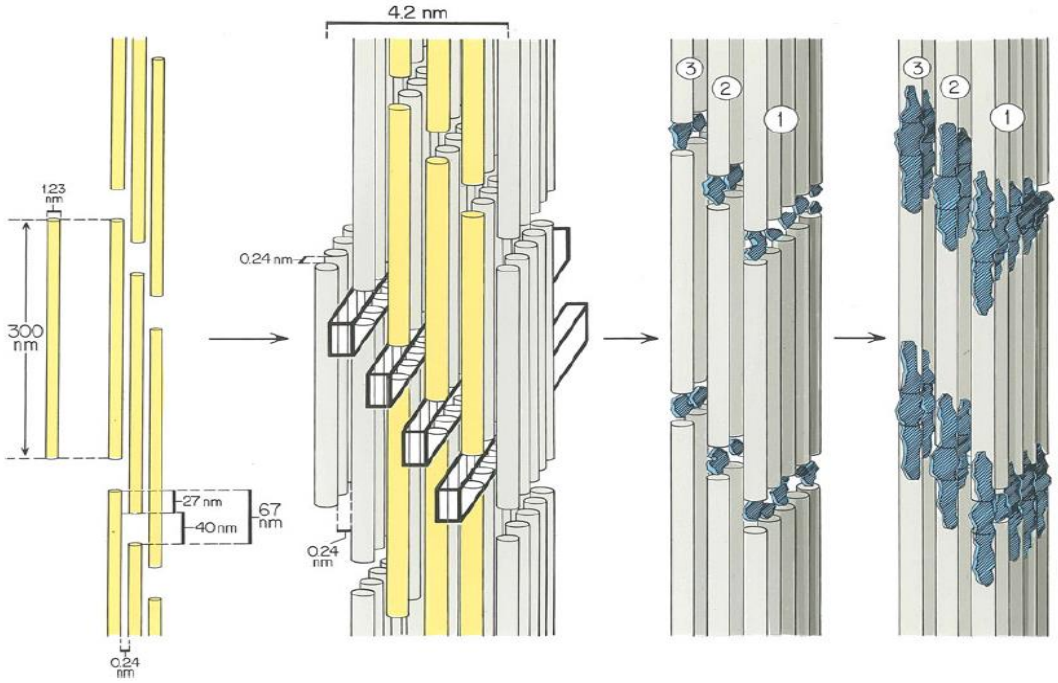



**Figure 2:** Collagen fibril organization and location of mineral formation[8]. Individual collagen molecules of approximately 300 nm organize into fibrils with a 67 nm D-periodicity in which a 40 nm gap zone exists. It is within this gap zone that hydroxyapatite crystals composed of calcium and phosphorous nucleate and grow.

Changes in the fibril structure have been speculated to influence intrafibrillar collagen mineralization in diseases such as osteogenesis imperfecta[9] but the impact of collagen fibril structure modulation by non-collagenous proteins (NCPs) have yet to be correlated with the quality and quantity of collagen mineralization in bone. In this study we elucidate how the NCP, Discoidin Domain Receptor 1 (DDR1), modulates bone mechanics and mineralization.

*Discoidin Domain Receptor 1*

DDR1 is a receptor tyrosine kinase that binds to collagen type I as its ligand[10]. DDRs are characterized by three distinct regions: the extracellular domain (ECD), a transmembrane region (TM), and an intracellular kinase region. A schematic of the three regions can be seen below in Figure 3.


**Figure 3:** Three General Regions of DDR proteins[10].

DDR1 is expressed as a membrane bound protein in several cell types including osteoclasts and osteoblasts[11]. The ECD of DDR1 is also shed as a soluble protein in the extracellular matrix[12]. The Agarwal laboratory has extensively characterized how DDR1 ECD modulates the collagen fibril structure by impacting the diameter and organization of the D-period in the fibril[13,14]. As a tyrosine kinase, DDR1 is also responsible for

downstream signaling events and cell behaviors[10] once it becomes phosphorylated upon binding to collagen[15,16].

DDRs have been linked to regulating collagen mineralization in pathological situations, with DDR1 expression showing a positive correlation with atherosclerotic calcification in an earlier study[17]. The Agarwal lab has also reported enhanced matrix mineralization in cells expressing the DDR1 ECD compared to control cells[13]. However, one conflicting study has recently reported enhanced mineralization by vascular smooth muscle cells lacking DDR1[18]. While these observations indicate that DDR1 modulates collagen mineralization, it calls for further investigations to understand how DDR1 modulates mineralization across various tissues in health and disease.

*Bone Mineralization and mechanical properties*

The hierarchical structure of bone consists of macroscopic features such as osteons and Haversian canals in compact bone and nanoscale features such as mineralized collagen fibrils[18]. The unique mechanical properties of bone are primarily derived from these mineralized collagen fibrils at the nano-structural level[18]. The collagen content has been associated with the toughness of bone and mineral content has been associated with the stiffness of bone[3,4]. This mechanical association has even been elucidated on the molecular level. Full-atomistic calculations in computer simulations have illustrated that an increase in mineral density is associated with an increase in the tensile modulus, or stiffness, and that collagen is more responsible for bone's deformation response[2]. While the collagen and mineral constituents of bone provide very

different mechanical properties in bone tissue, it is the molecular interactions of these two constituents that ultimately promote the formation of mineralized bone tissue[8].

*Research Goals and Motivation*

The research question that we aim to answer is if expression of DDR1 regulates mineralization of long bones in-vivo. Our current underlying hypothesis is that the modulation of the collagen fibril structure by DDR1 will inhibit the intrafibrillar mineralization of the collagen fibril present in the bone tissue. My individual contribution to this project is the analysis of biomechanical and mineral data from the femurs of DDR1 knockout (KO) and wildtype (WT) mice by utilizing three-point bending data and TGA/ICP-OES analytical techniques respectively. In particular, I focused on correlating the stiffness of the bone samples with the mineral content (Calcium and Phosphorous) of the bone samples to determine differences between KO and WT bone samples. This data, along with additional studies performed in the Agarwal laboratory, will be used to determine how the collagen fibril structure, when altered by DDR1, affects mineralization.

Studying and understanding the biomineralization of natural tissues, such as bone, is of great interest in the context of creating biomimetic materials that can be used for therapeutic purposes such as the regeneration of natural mineralized tissues[1]. Studying novel NCP's, such as DDR1, and elucidating its role in biomineralization will provide more comprehensive information regarding the in-vivo mineralization process that could have potential applications for biomimetic materials.

## Methodology

*Mechanical Testing*

Femurs were extracted from age (4 to 6 month) and gender matched KO and WT mice littermates after euthanasia via the PI's approved IACUC protocol 2008A232-R1. The femora were wrapped in PBS soaked gauze and stored at -20$^{o}$C until use. Three-point bending tests were performed in conjunction with Dr. Alan Litsky of the Biomedical Engineering Department on all right femurs to acquire mechanical data such as the stiffness, peak load, and the toughness of the bone samples.

These parameters were determined from the plots of load vs displacement data for each sample. The peak load was determined as the highest force applied to the bone before fracture of the bone occurred. The linear curve fitting capabilities of excel were utilized to determine the stiffness of each sample, which is the slope of the linear section of the load-displacement curve occurring just after loading begins and up till the point of fracture. Approximately 18-20 data points were chosen from this linear region to quantify stiffness. The toughness of the sample is described as the area under the load-displacement curve, which was determined using MATLAB software that numerically

estimates the area based on the trapezoidal rule. Figure 4 below details how raw load-displacement data were analyzed for the different biomechanical parameters.

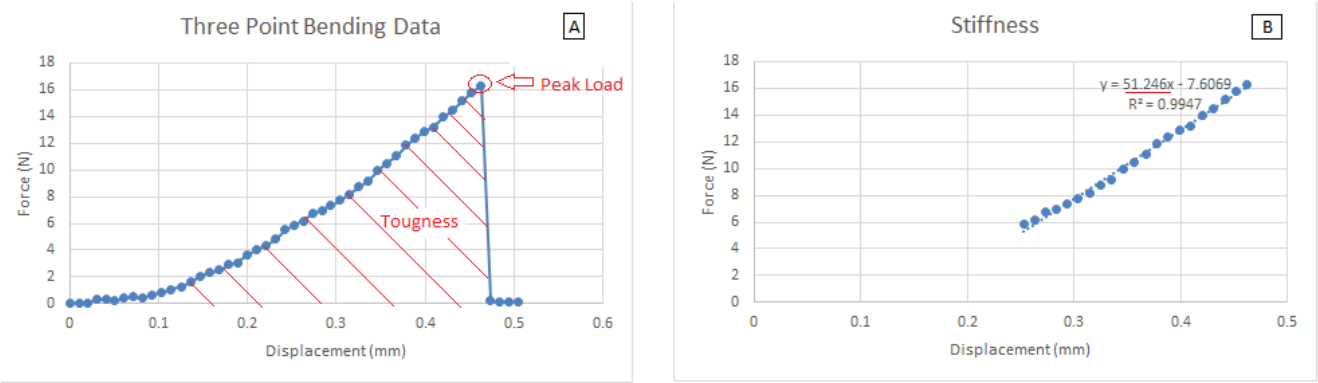



**Figure 4:** Determination of mechanical properties from three point bending data. Picture A details how peak load and toughness parameters where determined from the raw bending data collected from a sample. Picture B illustrates how a linear section of the raw bending data was used to determine the stiffness of the sample. The slope of the linear fit, underlined in red above, was used to approximate the stiffness of the sample.

After performing the mechanical tests, the broken bone samples were defatted and cleaned off of bone marrow. This process involved the physical removal of the excess tissue, soaking the bone samples in 2:1 and 1:2 chloroform/ethanol solutions for two consecutive days each and air drying. Then, one-half of the broken sample was subjected to Thermogravimetric Analysis (TGA) and ICP-OES protocols detailed below while the other half of the broken sample was utilized for Raman imaging protocols that are not included in with this thesis project.

*TGA Protocol*

A TA instrument's TGA Q50 (SN: 0050-0984) was utilized in conjunction with Dr. Karn in the OSU chemistry department to ash the bone samples in order to determine the relative mineral content of the bone sample on a percent mass basis. The principal behind this procedure is that the organic and other non-mineral components of the bone

sample will be burned up, leaving only the mineral content of the bone behind. This allows the mineral content of each sample to be discerned on a percent mass basis.

The protocol performed on each specimen consisted of three sequential heat ramps followed by a single isothermal hold. The first heat ramp increased the temperature of the furnace at a rate of 20$^{o}$C per minute to a target temperature of 100$^{o}$C. The next two heat ramps raised the temperature from 100$^{o}$C to 200$^{o}$C at a rate of 10$^{o}$C per minute and from 200$^{o}$C to 800$^{o}$C at a rate of 5$^{o}$C per minute respectively. An isothermal hold was then performed at 800$^{o}$C for an hour, making the total procedure run-time for each sample approximately 192 minutes. The pre-ashed weight of specimens used in this procedure ranged from 4.18 to 15.01 mg with an average mass of 10.64$\pm$ 3.03 mg. A sterilized platinum tray was used to suspend each specimen in the furnace.

*ICP-OES Protocol*

This analytical technique was performed in conjunction with the Trace Element Research Laboratory (TERL) at OSU, and was useful in determining the concentrations of Calcium (Ca) and Phosphorous (P) mineral in bone samples along with the Ca:P ratio. This technique utilizes inductively coupled plasma at high temperatures to produce the characteristic electromagnetic radiation of the target atom or ion. To prepare the bone samples for this technique, the ashed bone samples (after TGA analysis) were subjected to acid digestion in 5 ml of concentrated nitric acid by using a Parr Microwave Acid Digestion Bomb. The sample and acid bomb combination were placed in a microwave for 20 seconds to allow complete digestion. These preparations were done per specifications

in the Parr user manual concerning preparation of organic samples[20]. See the appendix for a diagram of the Parr Microwave Bomb that was used in this study.

The digested solutions were then diluted to 2:50 and 3:50 concentrations in purified water, providing two dilutions with which to calculate and compare mineral percentages. This allowed the percent Ca and P content in each sample to be determined by TERL using standard curves and ICP-OES equipment once ash weight was taken into consideration. However, correlating changes in the Ca and P content to decreases in bone mineral is not a traditionally accepted method since it is the combination of these two minerals that lead to hydroxyapatite formation[21,22]. Therefore, the Ca:P ratio was determined for each sample in order to obtain a metric that better characterized mineral quality.

*Statistical Analysis*

A student's two-tailed unpaired t-test was performed to ascertain statistically significant differences between samples for both mechanical and mineral parameters. A $p<0.05$ was regarded as significant.

## Results and Discussion

Biomechanical and percent mineral data were collected from male and female femurs of the DDR1 KO and WT mice. Data was compiled from 12 samples of the KO and WT genotype in the female biomechanical tests, and 11 KO and 14 WT samples in the male biomechanical tests. Percent mineral data was collected from six female mouse

pairs and five male mouse pairs using TGA. Additionally, ICP-OES analysis was performed on 4 female mouse pairs to elucidate the specific mineral percentages of Ca and P in ashed bone samples.

*Biomechanics and Percent Mineral Results*

A three-point bending analysis was used to determine the stiffness, peak load, and toughness of each sample. The compiled data for all mechanical tests excludes sample outliers (n=4) that were larger than 1.5 standard deviations away from the sample average for each parameter. Theses outliers could result due to variations in the positioning of the bone for the mechanical testing, which may have impacted the results. For example, in the bending tests, one side of the bone was subjected to tension forces and the other to pressure forces. If different areas of the bone respond differently to tension and pressure forces, then the results may have been affected. For future tests, it will be important to standardize which side of the bone, anterior or posterior, will experience these forces. This will help to minimize any possible changes in mechanics due to how different areas of bone may respond to tension or pressure forces.

The stiffness of each sample was of particular interest in this study since stiffness in bone is known to have a positive correlation with the amount of bone mineral. Figure 5 on the next page displays stiffness data for female and male KO and WT mice. Similar graphs for peak load and toughness can be found in the appendix.

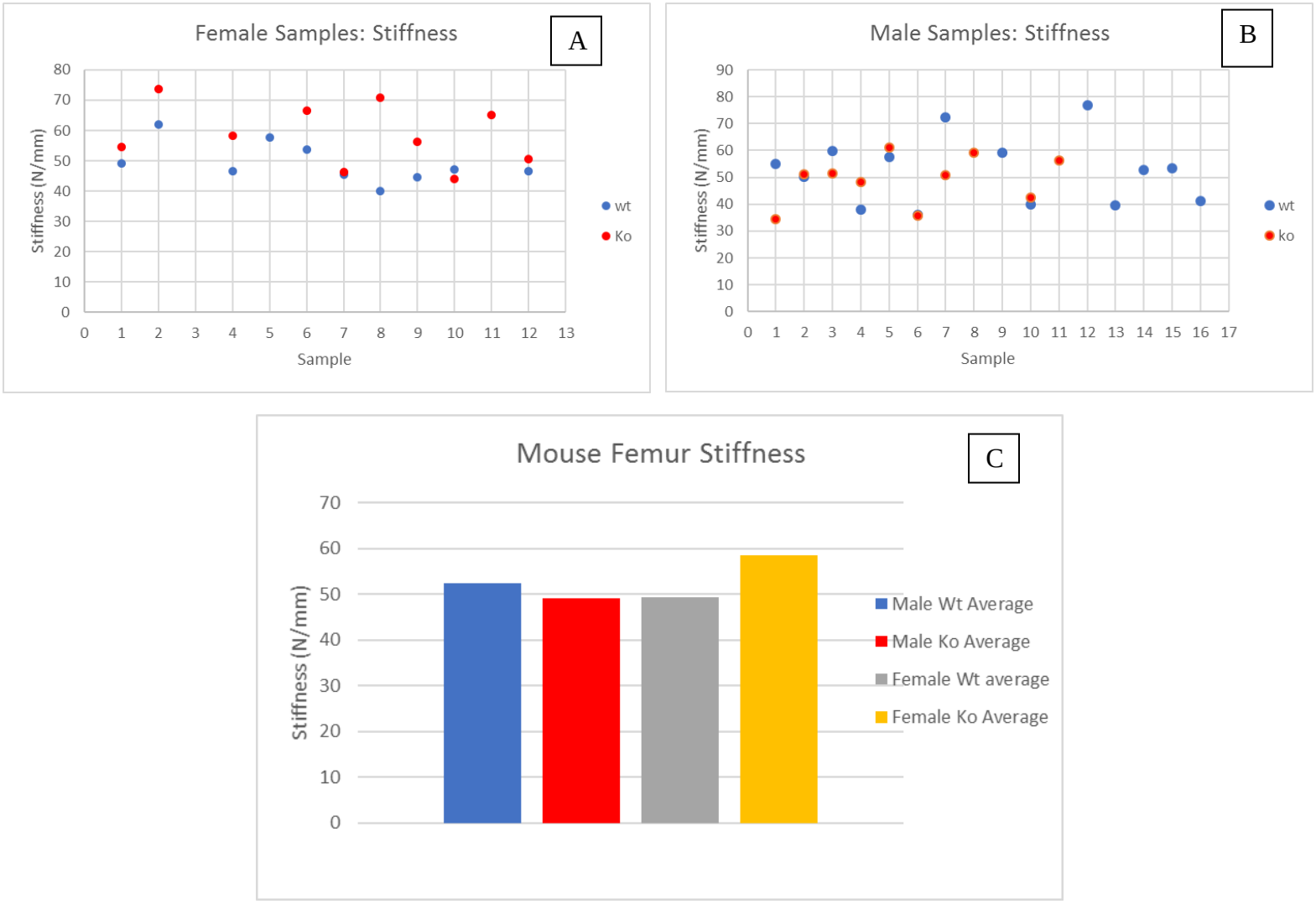



**Figure 5:** Stiffness of (A) female and (B) male specimens. for individual mice femora. (C) Bar graphs representing average stiffness for each gender and genotype. Female KO femora were stiffer than WT while the male femora showed no significant difference between the two genotypes.

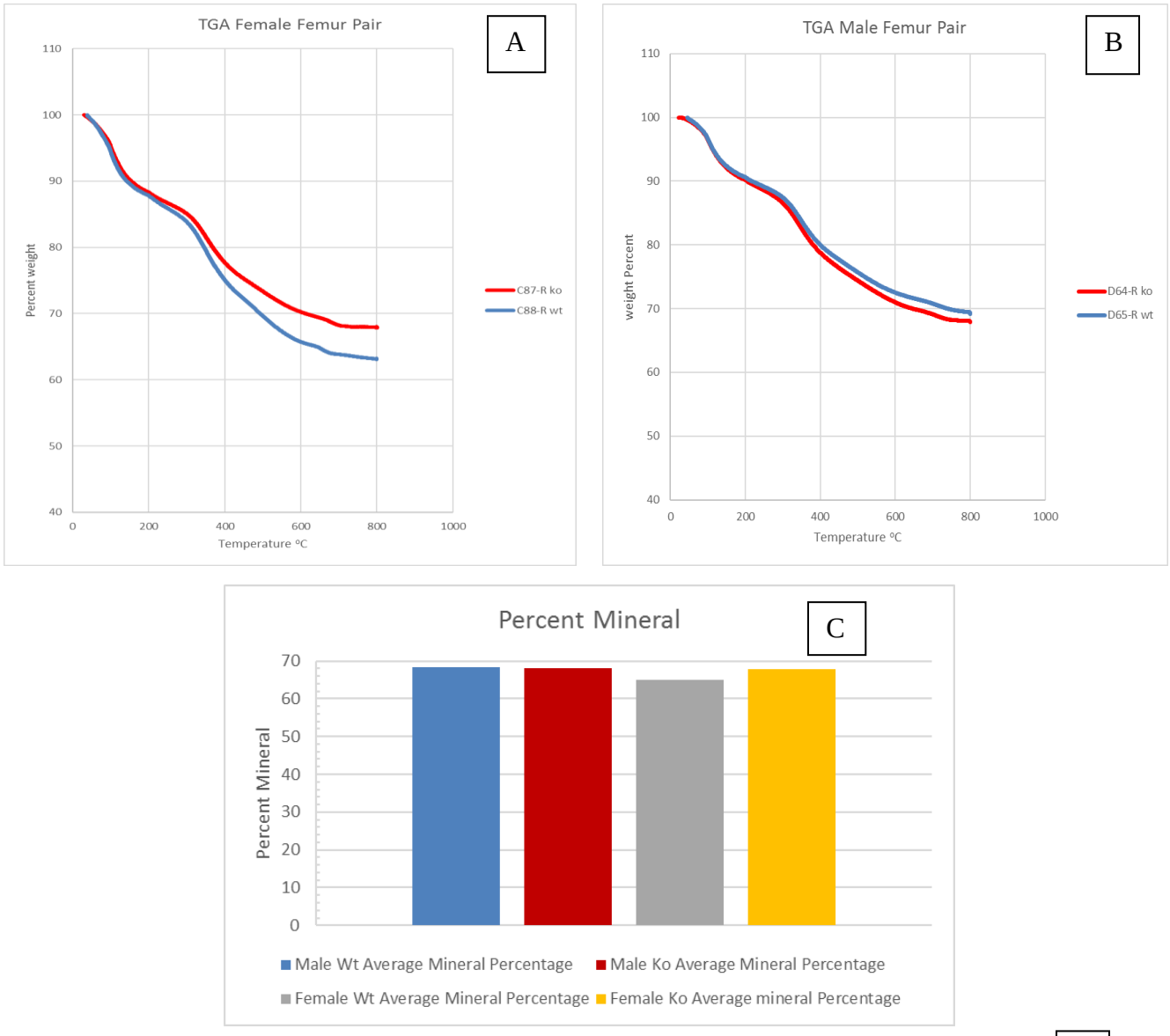
Different trends were observed in bone stiffness for male and female mice. Female KO mice had a higher average stiffness than WT mice (58.65 N/mm and 49.25 N/mm respectively), while male KO mice showed a trend towards lower stiffness than WT (49.10 N/mm and 52.30 N/mm respectively). A two-tailed t-test was performed and

determined a p-value of 0.025 for the stiffness of female mouse pairs and a p-value of 0.50 for the stiffness of male mouse pairs. A similar statistical analysis was performed for all mechanical parameters, but the stiffness parameter for female bones was the only mechanical parameter that showed statistically significant results. Table 1 below details the statistical analysis that was performed on all mechanical parameters.

**Table 1:** Mechanical parameters.

| 6 month old Femurs | Female Mouse data | | | Male Mouse Data | | |
|---|---|---|---|---|---|---|
| | Ko (Avg.) | Wt (Avg) | P-value | Ko (Avg.) | Wt (Avg.) | P-value |
| Peak Load (N) | 19.84 ± 2.55 | 18.44 ± 1.83 | 0.178 | 19.18 ± 2.08 | 20.39 ± 1.79 | 0.159 |
| Stiffness (N/mm) | 58.65 ± 9.64 | 49.25 ± 6.29 | 0.025* | 49.10 ± 8.62 | 52.3 ± 12.12 | 0.500 |
| Toughness (N.mm) | 4.13 ± 0.85 | 4.34 ± 0.47 | 0.515 | 5.33 ± 1.35 | 5.04 ± 1.15 | 0.606 |
| *denotes Significance | | | | | | |

Percent mineral in broken femora pieces were analyzed using TGA. The Mineral to Matrix ratio (MMR) was also determined based on comparing the percent mineral to the percent organic material. Figure 6 on the next page details results from TGA performed on female and male mice Femurs.




D

| 6 month old Femurs | Female Mouse data | | | Male Mouse Data | | |
|---|---|---|---|---|---|---|
| | Ko (Avg.) | Wt (Avg) | P-value | Ko (Avg.) | Wt (Avg.) | P-value |
| % Mineral | 67.80 ± 1.86 | 64.92 ± 3.61 | 0.154 | 67.98 ± 0.79 | 68.37 ± 0.46 | 0.425 |
| MMR | 2.12 ± 0.19 | 1.88 ± 0.26 | 0.132 | 2.13 ± 0.08 | 2.16 ± 0.05 | 0.431 |

**Figure 6:** Panels A and B show a comparison of typical individual tests for female and male respectively. The percentage present at 800°C was used to estimate the mineral content in a sample. Panel C compares the average mineral content across genders and genotypes and Panel D displays statistical analysis for TGA and MMR data. Opposite trends are once again seen in female and male mice, with female mice showing higher mineral content in KO variants and male KO mice showing nearly similar mineral content as WT.

The higher stiffness in female KO bones correlates with the higher mineral content in these bones whereas the mineral content in the male mice was slightly lower for the KO mice. However, statistical analysis did not reveal any significant difference between the mineral content of mice variants for either gender.

*Mineral Analysis*

Mineral analysis was performed on four pairs of female specimens utilizing ICP-OES to determine the percentage of Calcium (Ca) and Phosphorous (P) present in samples on a per mass basis. The TERL at OSU determined a concentration (in ppm) of Ca and P from two dilutions of acid digested ashed bone sample that had already been subjected to TGA. The known concentrations and dilution factors (2:50 and 3:50) were used to determine the mass and mineral percentages of Ca and P present in each sample based on its ashed weight and on its whole bone weight. The mineral percentages obtained from the two dilutions were averaged to give a single mineral percentage and Ca:P ratio for each tested sample (see appendix for more details). The results of the mineral analysis are summarized in Table 2 along with the bulk percent mineral data from the TGA results.

**Table 2:** Mineral Analysis

| 6 month old Femurs | Female Mouse data | | |
|---|---|---|---|
| | Ko (Avg.) | Wt (Avg) | P-value |
| % Mineral | 67.80 ± 1.86 | 64.92 ± 3.61 | 0.154 |
| MMR | 2.12 ± 0.19 | 1.88 ± 0.26 | 0.132 |
| % Ca (Ashed Bone) | 36.56 ± 2.63 | 40.21 ± 0.67 | 0.059 |
| % P (Ashed Bone) | 16.18 ± 1.08 | 17.88 ± 0.31 | 0.039* |
| % Ca (Whole Bone) | 24.70 ± 2.28 | 26.62 ± 0.68 | 0.213 |
| % P (Whole Bone) | 11.08 ± 1.00 | 11.84 ± 0.27 | 0.25 |
| Ca:P | 2.30 ± 0.07 | 2.23 ± 0.20 | 0.204 |
| *denotes Significance | | | |

It is interesting to note that WT mice have a larger portion of their mineral percentage composed of both Ca and P than KO mice variants, suggesting that additional elements would account for the higher mineral content in KO bones. However, the Ca:P ratio is a standardized representation of the amount of hydroxyapatite contained within the bone that takes into account both Ca content and P content[21]. The Ca:P ratio was very similar for KO mice and WT mice with a ratio of 2.30 for KO mice compared to the WT ratio of 2.23. In studies conducted on similar murine models, such as rabbits and rats, it was found that Ca:P ratio's for osteoporotic femora were closer to 1.82 and 1.29 respectively where as healthy models of rabbit femur had a ratio between 2.08 and 2.17[21,22]. These ratios very likely indicate that the bones from both genotypes in this study are physiologically normal and healthy since these ratios are comparable to the normal states of bones in similar murine models and are higher than the Ca:P ratios associated with the diseased states of these models. These earlier studies utilized X-ray absorptiometry, AES, and EDX imaging techniques to determine the Ca:P rather than techniques such as AAS or ICP-OES.

It should also be noted that the methods used for quantifying Ca and P in this study, especially concerning sample preparation, are more novel in scope. Other studies have used acid digestion[23] to prepare samples for mineral measurements, but these preparations were performed over longer time intervals, with the samples hydrolyzing overnight rather than using a microwave. It is unknown whether our faster approach to acid digestion would impact the results compared to the gentler acid digestion procedures that have been used.

## Conclusion

This study examined the bone mechanics and mineralization of DDR1KO and WT male and female femurs. A higher stiffness was observed for DDR1 KO female mice which correlated with its greater percent mineral in KO mice compared to WT mice. No significant differences were observed in the Ca:P ratio in mouse bones, indicating that the bones were within the range of physiologically normal bones and similar in quality across the two genotypes. Our results are consistent with earlier reports, where a positive correlation in stiffness has been observed in increased mineral content[2,3]. Our results also show a gender dependence in the mechanical and material properties of mice DDR1 KO mice femora. These results merit additional research and investigation into why gender differences exist between male and female DDR1 KO and WT mice since there are currently no conclusive explanations explaining the cause of these gender differences.

Further research studies need to be conducted to fully elucidate the role of DDR1. Examples of such studies include studies that look at mice in different stages of development. This study looked at mice around 6 months of age, but perhaps older mice closer to 12 months of age would show more significant trends with regards to DDR1. Additional mechanical parameters could also be analyzed, such as parameters related to the rotational integrity of the bone. Further studies on evaluating DDR1 expression, collagen content and mineralization in bone diseases could help elucidate the role of DDR1 in modulating pathological remodeling.

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# Appendix

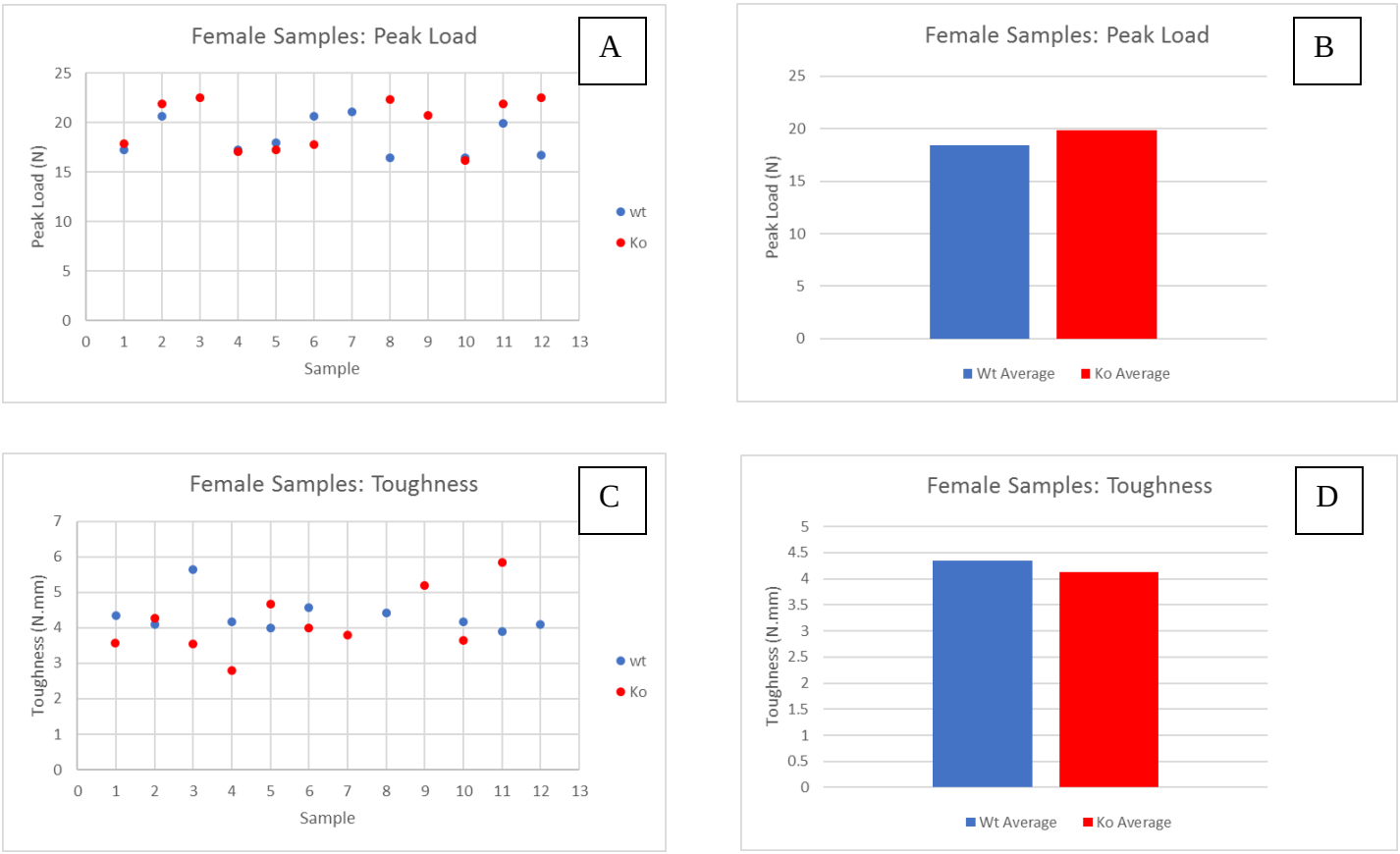


**Figure A1:** Female mechanics data: peak load and toughness. Pictures A and C compare individual samples wtihin 1.5 standard deviations of the sample average and pictures B and D compare the average vales for peak load and toughness of KO and WT mice variants.

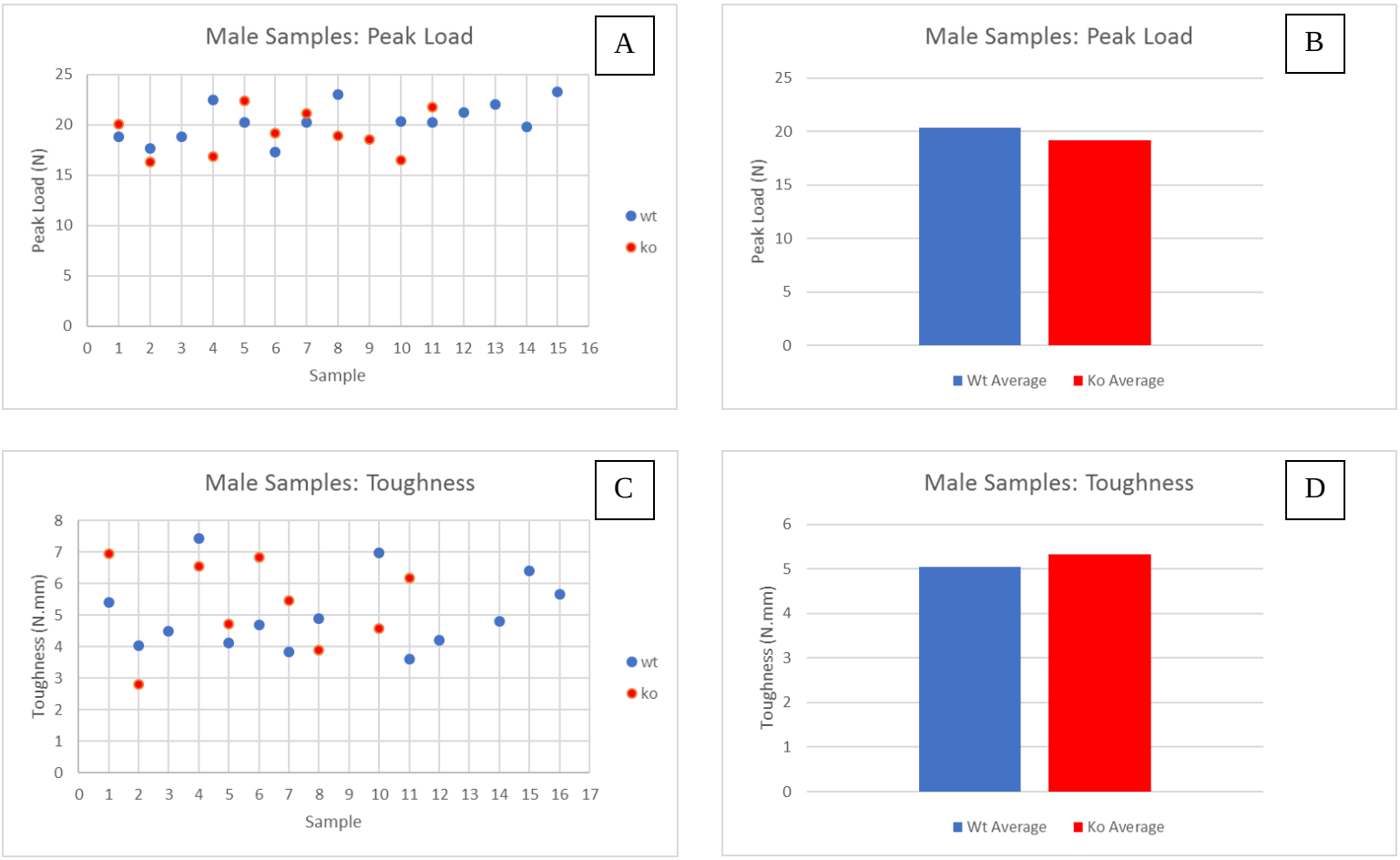


**Figure A2:** Male mechanics data: peak load and toughness. Pictures A and C compare individual samples within 1.5 standard deviations of the sample average and pictures B and D compare the average vales for peak load and toughness of KO and WT mice variants.

**Table A1:** KO sample's ICP-OES mineral analysis. This table details how calcium and phosphorous mineral percentages were determined as the average of two separate sample dilutions. The standard deviation between the two dilutions is also provided

| Ko Samples | C71-R 2:50 | C71-R 3:50 | STDV | C87-R 2:50 | C87-R 3:50 | STDV | D23-R 2:50 | D23-R 3:50 | STDV | D27-R 2:50 | D27-R 3:50 | STDV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| % Ca Ash weight | 39.02 | 37.92 | 0.7778175 | 37.69 | 37.80 | 0.077782 | 35.896 | 28.17 | 5.463107 | 39.95 | 36.03 | 2.7718586 |
| **% Ca Average** | **38.47** | | | **37.745** | | | **32.033** | | | **37.99** | | |
| % P Ash weight | 17.44 | 16.92 | 0.3676955 | 16.88 | 17.17 | 0.205061 | 16.24 | 12.72 | 2.4890159 | 15.95 | 16.11 | 0.1131371 |
| **% P Average** | **17.18** | | | **17.025** | | | **14.48** | | | **16.03** | | |
| Ca:P | 2.239 | | | 2.22 | | | 2.36 | | | 2.37 | | |

**Table A2:** WT sample's ICP-OES mineral analysis. This table details how calcium and phosphorous mineral percentages were determined as the average of two separate sample dilutions. The standard deviation between the two dilutions is also provided

| Wt Samples | C84-R 2:50 | C84-R 3:50 | STDV | C88-R 2:50 | C88-R 3:50 | STDV | D25-R 2:50 | D25-R 3:50 | STDV | D28-R 2:50 | D28-R 3:50 | STDV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| % Ca by weight | 41.16 | 39.58 | 1.1172287 | 41.56 | 40.02 | 1.088944 | 40.87 | 40.290 | 0.4101219 | 39.19 | 38.97 | 0.1555635 |
| **% Ca Average** | **40.37** | | | **40.79** | | | **40.58** | | | **39.08** | | |
| %P Ash weight | 18.3 | 18.04 | 0.1838478 | 18.37 | 17.99 | 0.268701 | 17.97 | 17.490 | 0.3394113 | 17.43 | 17.46 | 0.0212132 |
| **% P average** | **18.17** | | | **18.18** | | | **17.730** | | | **17.445** | | |
| Ca:P | 2.22 | | | 2.17 | | | 2.288776086 | | | 2.24 | | |

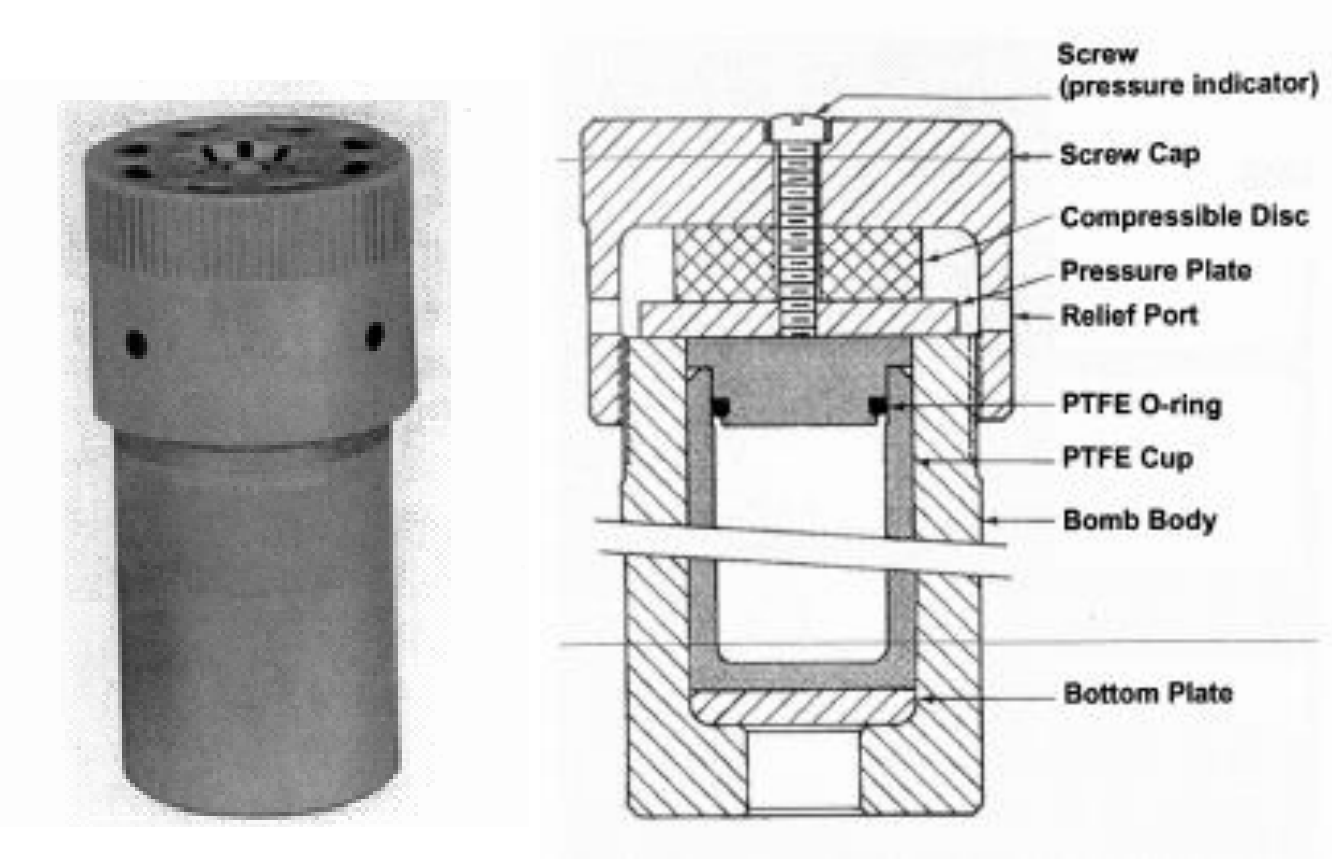


**Figure A3:** Parr Microwave Acid Digestion Bomb[20]. This product was used along with high concentration nitric acid to digest the ashed bone samples in preparation for ICP-OES. The sample with nitric acid was contained inside the PTFE cup.